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Title: The regulation of lymphocyte activation and proliferation

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Abstract

Activation induced proliferation and clonal expansion of antigen specific lymphocytes is a hallmark of the adaptive immune response to pathogens. Recent studies identify two distinct control phases. In the first T and B lymphocytes integrate antigen and additional costimuli to motivate a programmed proliferative burst that ceases with a return to cell quiescence and eventual death. This proliferative burst is autonomously timed, ensuring an appropriate response magnitude whilst preventing uncontrolled expansion. This initial response is subject to further modification and extension by a range of signals that modify, expand and direct the emergence of a rich array of new cell types. Thus, both robust clonal expansion of a small number of antigen specific T cells, and the concurrent emergence of extensive cellular diversity, confers immunity to a vast array of different pathogens. The *in vivo* response to a given pathogen is made up by the sum of all responding clones and is reproducible and pathogen specific. Thus, a precise description of the regulatory principles governing lymphocyte proliferation, differentiation and survival is essential to a unified understanding of the immune system.

Introduction

According to classic two-signal theory, lymphocytes face a binary decision when stimulated by antigen and must choose between tolerance (death) and activation (proliferation). A second signal is needed to tip the balance from one state to the other. Careful studies of the control of T and B cells are substantially modifying this view and replacing the binary decision with a quantitative signal integration model that tempers the overall strength and type of response to the nature of the threat. As a result, the magnitude and duration of the immune response must be seen as continuously variable. How this T cell behaviour is modulated, at molecular, single cell and population levels to achieve such a rich set of alternative outcomes remains under intensive investigation. **Figure 1** illustrates the control of lymphocyte proliferation as a two-stage process with each naive cell integrating activation signals, stochastic probabilities and ongoing signals to control the rich heterogenous population outcome.

Early programming cooperates with ongoing signal integration to control the response magnitude

In reviewing progress to date, it is helpful to distinguish two separate stages for lymphocyte activation. In the first, an autonomously programmed response, leading to multiple changes in

fate are motivated by the initial stimuli. T cells divide several times subsequent to removal of stimuli. In CD8+ T cells this can be after a very brief initial exposure [1-4] whereas in CD4+ T cells a longer antigen exposure is required to commit cells to an autonomous proliferative burst [5,6]. A similar initial autonomous clonal division is observed in B cells [7-9]. In the second phase of control, the early cell programming is further modified by ongoing signals. For example, T cells modify their own environment by producing the growth factor IL-2 that can promote their continued division [10]. As signalling inputs can operate simultaneously on both phases of the response it can be difficult to determine individual control mechanisms. Costimulatory signals as well as cytokines and chemokines all play fundamental roles in regulating one or more parameters that determine the final cell numbers. Additional features of signal control, such as increased TCR affinity, enhanced dose, duration or mechanical properties of TCR-pMHC contacts or prolonged antigen exposure also result in a greater response magnitude, with increased rounds of cell division or greater recruitment of cells into division [11,12]. In many cases, the early expansion rate of activated T cells is unchanged in these systems. Instead, the duration of their expansion is increased [2,11,12] suggesting an equal proliferation rate of activated cells with more cells dropping out of division or dying sooner under weak stimulation conditions.

For T cells the extent of the initial proliferative burst is a major determinant of response magnitude *in vitro* and *in viv*o. The average number of divisions undergone can vary with stimulation. T cells integrate all the signals they receive through the TCR, costimulatory molecules and cytokines receptors to determine the size of the initial burst, referred to as their initial 'division destiny' (DD) [13]**. Multiple contributions to DD, provided at the same time, added arithmetically allowing predictions to be made for the final response. In this study the authors demonstrate that many different combinations of costimulatory signals are capable of adding to a significant response outcome. These experiments also highlight control of the second phase of the T cell response: Cytokines such as IL-2 and IL-4 play both a major role in initiating, as well as sustaining / extending cell division beyond their initial autonomous DD. This maintenance was shown to be particularly important for T cells that have migrated to sites of infection or inflamed tissue [14,15]. T cells modulate IL-2 production and integration of IL-2 signals as a mechanism of paracrine communication in order to fine tune and optimise the response magnitude [10,16,17]. Furthermore, although the proliferative effect of a particular stimulus can act predominantly on initial programming, it may also have alternate modulatory roles during the subsequent progression of the response. For instance, CD28 signalling

increases IL-2 production and sensitivity [18]. If IL-2 is blocked, the CD28 signal is still effective at programming and promoting division destiny changes into naïve T cells but the signal must be received prior to the first division to have an effect [13]. Later engagement of CD28 alters downstream fate selection during the response without having any further proliferative impact [19].

The regulatory precision of the initial burst of proliferation and return to quiescence by stimulated T and B cells [9] suggested a common mechanism might be found. This proved correct. The cell division-promoting proto-oncogene Myc [20]** is induced upon activation and lost over time until a minimal threshold is crossed, and division ceases. Rather than diluting by division, as was expected, Myc levels degraded over time at a predictable rate that was faithfully passed on to daughter cells independently of division number. As expected for this form of control, the level of Myc protein induced after activation was found proportional to the strength and number of signals received by the cell, and its level highly correlated with subsequent DD. Therefore, Myc translates the signals the cell receives into the time that each founder cell is given to divide before returning to quiescence [20].

Furthermore, in addition of providing stimulatory signals on their own, inflammatory signals such as IL-12 and IFN-α can increase sensitivity to IL-2 signalling [14,21,22]. Continuous signalling via IL-2 or other cytokines slows the loss of Myc protein and therefore extending the period of time for which the cells can divide [20] [23]* [24]. Understanding how these factors function as part of a subcellular network, combining and cooperating to determine the ultimate proliferative potential of an individual T cell remains a major objective in the field of lymphocyte biology.

These studies may have a further counterpart in the germinal centre. During affinity maturation germinal centre B cells travel from the light zone where they undergo positive selection to the dark zone and undergo somatic hypermutation [25]. Interaction with Tfh cells during the positive selection process provides proliferation and survival signals. In this context Myc expression is induced through interaction with Tfh cells and is seen in a proportion of, presumably, recently activated cells [26,27]. Affinity dependent stimulation is thought to control proliferation and survival with low affinity B cells dying in the light zone while dysfunctional BCR induced through mutation leads to cell death in the dark zone [28,29] [30]*. In parallel with the control of T and B cell DD, it seems likely that higher affinity B cell clones receive stronger stimuli and accumulate more Myc, extending their duration of proliferation in

the dark zone and therefore licensing these clones for more extensive somatic hypermutation [29].

Clonal concordance, probabilistic events and fate inheritance shape the response

The proliferative and phenotypic profile of a responding T cell population is highly heterogeneous, even under highly controlled *in vitro* conditions. Despite this extensive diversity, there is a remarkable concordance of the proliferative fate within a clonal family while a considerable disparity is observed between different clones even in response to identical stimulation conditions [31] [32]* [33]*. *In vivo* studies have also demonstrated extensive heterogeneity in clone size and a distinct correlation between clonal proliferative potential and cellular phenotype [34-39]. The phenotypic correlation with clonal burst size points to a role for both heritability and division as determinants of the emergence of cellular heterogeneity [35,36,40]. This is complemented by studies that have elucidated a role for division progression in regulating specific components of T cell differentiation, such as cytokine production, cytotoxicity and surface marker expression [40-45].

Although there is a clear influence of clonal membership on division progression and phenotype, *in vivo* studies demonstrate substantially greater intraclonal diversity than the striking concordance observed *in vitro* [32,37,38]. TCR signal quality strongly determines the response outcome on a population basis, and although weakly stimulated cells expand less, many are still able to acquire effector functions and differentiate into memory cells [11,46,47]. Therefore the fate of T cell clones is not controlled by the TCR ligation quality alone, but TCR ligation works in concert with quantitative integration of additional signalling and stochastic events to determine the fate outcome of the clonal progeny [13,33,35] [46]** [48] [49]* [50,51].

Many of the above studies highlight early stochasticity and familial heritability as key drivers of emergent heterogeneity. Several different models have been proposed to explain the diversity observed in T cell fates. The concept of asymmetric division of the founder cell resulting in two distinct fate outcomes of the daughter cells and their progeny has been proposed as a determinant of T cell fate and population heterogeneity [5,52-54]. Recent studies have proposed a role for the polarised segregation of Myc and subsequent asymmetric inheritance of metabolic programming as a determinant of CD8+ T cell fate selection [55,56]. However, the uneven inheritance of Myc between first-division T cells is at odds with its role

as a regulator of the highly symmetrical clonal phenomenon of DD [20]. In an alternative model the strong familial concordance in concert with early stochasticity are sufficient to describe the emergence of the clonal diversity [46,57]. Computational descriptions of clonal heterogeneity have also highlighted the capacity to resolve patterns of T and B cell diversification without the requirement of asymmetric fate segregation [50,58]. Furthermore, impairment of the capacity of cells to polarise their contents does not hinder the generation of lymphocyte diversity [59].

Recruitment into division as a first step directing the response magnitude

The number of cells recruited into an immune response is another key determinant of response magnitude. Two main factors determine the number of cells recruited into division: firstly, the induction of a new survival program driven by the strength of stimulation selecting for strongly activated cells to survive [60]. Secondly, whether the surviving cells reach the activation threshold to enter proliferation. On a single cell level this threshold is controlled by the sum of TCR affinity and dose [12] [61]* [62,63] and other signals received by the cells. Consistent with the mechanism of signal addition, IL-2 or additional costimulation increases this precursor frequency and promotes the entry of weakly stimulated cells into division [61,64-66]. Similarly stimulation through the costimulatory receptor CD27 lowers the affinity threshold required for activation, recruiting more low affinity clones into the response, potentially as a measure to broaden the subsequent memory pool repertoire [67].

On a population level, whether a response is observed is determined by the sum of all individual outcomes and the complex interaction between these cells and the molecules they produce. This can be described as a collective decision made by the T cell population [68]. IL-2 produced by strongly activated cells plays a critical role in this activation phase, as some low affinity T cells can reach the threshold to enter division through integration of IL-2 produced by strongly activated cells. The response rate of a mixed population of high and low affinity cells can be modelled and predicted accurately using a dynamic system incorporating IL-2, IL2R and PIK3 levels controlling the accumulation of Cyclin D to reach the threshold of cell cycle entry [69]** demonstrating how the signal integration by individual cells controlling their fates fine tunes the overall response on the population basis.

Survival as an additional and independent mechanism shaping the immune repertoire

The survival of activated T cells critically underpins the ability to form an immune response. This process is carefully regulated by a quantitative balance between pro- and anti-apoptotic

members of the Bcl-2 family proteins of the intrinsic apoptotic pathway (reviewed in [70]). A survival program is induced after T activation, that is distinct from their naïve survival program and operates simultaneously but independently to the proliferation program [71,72]. Both pro-survival and pro-apoptotic proteins are induced by T cell activation signals through the TCR, costimulatory molecules (i.e. CD28) or cytokines such as IL-2 [60,73-75]. A similar quantitative switch in survival programs occurs in B lymphocytes [70,76]. In cells receiving strong signals this balance favours a pro-survival state, however low affinity populations are more sensitive to shaping through differential survival as stimulation with a lower affinity TCR or a reduction in costimulatory signals and cytokines, favours death and the elimination of weaker responders [75,77-79]. This points to a key function in the regulation of the response quality.

As the lymphocyte survival program is initiated by many of the same signals as proliferation it is often difficult to distinguish the relative importance of contributions of these processes in shaping the immune response; however several lines of evidence suggest their regulation is independent, and can be uncoupled. For instance, CD28 signalling and other growth factors promote survival in the absence of proliferation [80]. CD8+ T cells deficient in the kinase Erk2 [81] or the transcription factor Bach2 [82] have been shown to have no defect in proliferative potential, but have a reduced response magnitude due to impaired survival. Furthermore the rate and extent of clonal expansion in strongly stimulated cells is not greatly impacted by cell death. When pro-apoptotic molecule Bim is deleted or pro-survival molecule Bcl-2 is overexpressed in T or B lymphocytes they undergo the same number of divisions in response to a given stimulus irrespective of the enhanced cell survival [13,20,83,84]. A similar effect can be observed in vivo, with Bim-deficient CD8+ T cells expanding to the same extent but taking longer to contract [85], further demonstrating the independence of cell division and survival. The significance of this is highlighted by the consequences of changes to either parameter. The combined effect of small changes in survival or DD time synergise to a greatly enhanced response when applied in combination and can be predicted by combining the probability distributions for each timer [20].

Conclusions:

When the number of alternative cell fates, and the large number of known modifiers are enumerated, the regulation of T and B cell responses appears impossibly complex. When combined with the fine detail of cellular niches in different tissues, and the potential for

transient and persistent signal exposure, it is easy to imagine a daunting combinatorial problem for control. This pessimistic view is at odds with the general observation that immune responses are typically robust and reproducible, and many features of cell responses can be recreated in much simpler *in vitro* environments.

In resolving this paradox, we suggest that a timed cellular program, that includes an automated return to quiescence and even eventual death, can serve as a powerful new paradigm for interpreting the complex control of T, B and GC cell responses. Manipulation of this core cellular program by multiple modifying inputs provides the foundation for building a reproducible, but highly regulated system. Such a model can also help explain how the present complex system may have evolved from more primitive developmental states that utilised a higher level of cellular autonomy to affect an adaptive immune outcome [7]. We envisage that continued work on this activation paradigm with increasingly quantitative tools will deliver scalable models with the power of prediction and significant potential for immune system control.

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Figure legends

Figure 1. An autonomous self-limiting response underlies T and B cell activation. All lymphocytes receive and process a large number of signals from their environment, antigen and accessory cells. These signals serve to set timers for the burst of division and the eventual death of these cells, as well as either division-linked or timed differentiation changes. Similar cells do not perceive signals in identical manner, leading to clonal family dependent variation that may have a stochastic basis (1). All cell functions governing this initial internal immune program can be affected by external signals, and further fate changes regulated by division or time ensures sensitive and broad ranging fate control (2, 3).